Effects of electroacupuncture at Taichong (LR 3) and Baihui (DU 20) on cardiac hypertrophy in rats with spontaneous hypertension

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Supported by Beijing Natural Science Foundation: Regulating effect of electroacupuncture on cardiac hypertrophy of spontaneously hypertensive rats based on PI3K/AKT signal transduction pathway (No. 7162121) /Young Teacher Program of Beijing University of Chinese Medicine: Mechanism of acupuncture on left ventricular remodeling in spontaneously hypertensive rats based on microRNA-195 targeting TGF/Smads signaling pathway (No. 2017-JYB-JS-030)

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Telephone: +86-15611525882
Accepted: April 28, 2019

Abstract

OBJECTIVE: To investigate the effects of electroacupuncture (EA) at Taichong (LR 3) and Baihui (DU 20) on myocardial hypertrophy in spontaneously hypertensive rats (SHRs).

METHODS: Thirty-six SHRs were randomly assigned to model, EA, and Losartan groups, with twelve rats per group. Twelve Wistar Kyoto rats were selected as the normal control group. Systolic blood pressure (SBP) and cardiac function were measured in all rats. Expression levels of factors associated with the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway were evaluated by western blotting and real-time PCR. Pathological changes of the heart tissue were observed by hematoxylin-eosin staining.

RESULTS: After treatment, enhanced SBP was significantly decreased in the EA and Losartan groups compared with the model group (P < 0.01). Echocardiographic and morphological analyses revealed that enhanced end-diastolic interventricular septal thickness and left ventricular posterior wall thickness, as well as ratio of left ventricular weight to body weight were markedly diminished in the EA and Losartan groups (P < 0.01 or P < 0.05), while reduced left ventricular end-diastolic dimension and left ventricular ejection fraction were significantly ameliorated (P < 0.01). Real-time PCR and western blotting analyses showed that the expression levels of PI3K, Akt, and mTOR in SHRs were significantly up-regulated by EA and Losartan (P < 0.01), while the expression levels of PTEN and ANP were down-regulated (P < 0.01).

CONCLUSION: EA at Taichong (LR 3) and Baihui (DU 20) inhibited the development of cardiac hypertrophy and improved the cardiac function in SHRs, possibly through regulation of the PI3K/Akt/mTOR signaling pathway.

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Keywords: Electroacupuncture; Rats, inbred SHR; Hypertrophy; Phosphatidylinositol 3-kinases; Proto-oncogene proteins c-akt; Mammalian target of rapamycin
INTRODUCTION

Left ventricular hypertrophy (LVH) is an important symptom in the development of hypertension, a leading cause of heart failure. LVH is considered to result from complex interactions of several hemodynamic and non-hemodynamic variables. The phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is an important signalling pathway that regulates a wide range of cellular processes, including proliferation, growth, apoptosis, and metabolism. Regulation of the PI3K/Akt/mTOR signalling pathway was reported to be effective for improving the supply of energy and increasing the contractility of myocardial tissue. Because of its convenience and relatively few side effects, acupuncture is widely used in the treatment of hypertensive myocardial hypertrophy. Electroacupuncture (EA) was shown to effectively lower blood pressure and inhibit the development of myocardial hypertrophy. EA was also reported to regulate the PI3K/Akt/mTOR signalling pathway. The present study aimed to determine whether the anti-hypertrophic effects of acupuncture are related to the PI3K/Akt/mTOR signalling pathway. To achieve this goal, spontaneously hypertensive rats (SHRs) were used as a model of spontaneous hypertension, and the effects of EA at Taichong (LR 3) and Baihui (DU 20) were evaluated.

METHODS

Animal preparation

Thirty-six SHRs and twelve Wistar Kyoto (WKY) rats were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China; License number: SCXK (Beijing) 2012-0001). All rats were specific pathogen-free, male, 12 weeks of age, and 220-260 g in weight. During the study, the rats were maintained in a controlled environment with temperature of (24 ± 1) °C, humidity of 60% ± 5%, and 12-h/12-h light/dark cycle, and received a standard diet and water ad libitum. All procedures for animal experiments were conducted in accordance with the World Health Organization International Guiding Principles for Biomedical Research Involving Animals and were approved by the Animal Care and Use Committee of Beijing University of Chinese Medicine (Beijing, China; Permit No. BUCM-4-2017121201-4022). All efforts were made to minimize animal suffering and euthanasia during the experimental process.

Animal grouping and intervention

The WKY rats were used as a normal group (n = 12). The SHRs were randomly divided into a model group (n = 12), EA group (n = 12), and Losartan group (n = 12) using a random number table. Sterilised disposable stainless-steel needles (0.18 × 13 mm; Hanyi TCM Co. Ltd., Beijing, China) were inserted 1-2 mm into the Baihui (DU 20) (located on the top of the head at the intersection of the middle sagittal line and the connection of the two ear apexes) and bilateral Taichong (LR 3) (located on the dorsum of the foot, in the depression anterior to the junction of the first and second metatarsals) acupuncture points of the rats in the EA group. After the insertion, Hans Acupoint and Nerve Stimulators (LH200H; Ji Sheng Medical Technology Co., Nanjing, China) were connected to the Baihui (DU 20) and right Taichong (LR 3), and electrical stimulation (2/15 Hz, 1 mA) was performed at 20 min/d for 30 d. The rats in other groups were immobilised in the same restrainer for 20 min without EA intervention. The rats in the Losartan group received a gavage of Losartan (Merck Sharp & Dohme Ltd., Pty, Australia) at 30 mg/kg per day, while the rats in the other groups received a gavage of water at equal volume.

Measurement of blood pressure

Before blood pressure measurements, the rats were prewarmed in a comfortable and ventilated environment of 36°C for 15 min. When the rats were quiet and calm, the systolic blood pressure (SBP) in the caudal artery was measured with a non-invasive blood pressure instrument (BP-2010A; Softron Biotechnology Co. Ltd., Beijing, China). The SBP of each rat was measured three times consecutively and the mean value was calculated. Blood pressure measurements were taken at 1 day before the intervention and on days 6, 12, 18, 24, and 30 after the intervention.

Echocardiographic analysis

After the 30-day intervention, all rats were anesthetised with 1% pentobarbital sodium, and their structural and functional changes were measured using a High Resolution Imaging System (VEVO ™ 2100; Visual Sonics, Toronto, Canada). The end-diastolic interventricular septal thickness (IVST), left ventricular posterior wall thickness (LVP-WT), left ventricular end-diastolic dimension (LVEDD), and left ventricular ejection fraction (LVEF) were recorded for three consecutive cardiac cycles and the mean values were calculated.

Histological measurement

The body weight (BW) of the rats was measured and recorded before sampling. After chest opening, the heart was washed with 4°C saline and dried with clean filter paper. The heart was then placed on ice and the large vessels and surrounding tissues were removed. The left ventricle was removed along the interventricular septum and the left ventricular weight (LVW) was measured. The ratio of LVW/BW was calculated as the left ventricular mass index (LVMi) to evaluate the level of hypertrophy. The left ventricles of six rats per group were randomly selected, immersed in 4% paraformaldehyde (Biosharp Co. Ltd., Hefei, China) for 24 h, and cut transversely at the midventricular level for paraffin sectioning. Subsequently, 4-μm myocardial sections were
cut transversely at the midventricular level, deparaffinised, rehydrated, and stained with haematoxylin and eosin (HE; Servicebio Co. Ltd., Wuhan, China). Images were captured with a digital camera connected to a microscope (BX53; Olympus, Tokyo, Japan).

**Real-time PCR and Western Blotting**

The six remaining left ventricles in each group were frozen in liquid nitrogen and stored at -80°C for real-time PCR and western blotting analyses. Total RNA was reverse-transcribed to cDNA using a TIANScript Reverse Transcription Kit (Tiangen Biotech, Beijing, China). A quantitative real-time PCR method was employed to detect gene expression levels using an ABI7500 Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) with Power SYBR Fast qPCR Kit Master Mix (2× Universal (KAPA Biosystems, Charlestown, MA, USA). NCBi-Primer software (National Center for Biotechnology Information, Bethesda, MD, USA) was used to design appropriate primers (Table 1) and GAPDH was employed as an internal reference. The amplification conditions are shown in Table 2. After the experiment, RQ Manager 1.2.1 software (Applied Biosystems) and Data Assist V3.0 software (Applied Biosystems) were used to calculate Ct values. The 2-aact method was used for relative quantification of the gene expression levels of PI3K, Akt, mTOR, phosphatase and tensin homolog deleted on chromosome ten (PTEN), and atrial natriuretic peptide (ANP).

Heart tissue was lysed in protein lysis buffer containing phosphatase and protease inhibitors (Youkezhuoey BioTech, Beijing, China). The protein concentration in the supernatant was determined by the BCA method and adjusted with RIPA buffer (1M tris-Cl 10 mL, 1M NaCl 30 mL, tritonx-100 2 mL, Sodium deoxycholate 0.25 g, 10% SDS 0.5 mL, 1M DTT). Equal amounts of total protein were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were then washed with TBST, incubated with goat anti-rabbit IgG (H + L) HRP (1:10000; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 40 min at room temperature, and washed with TBST for 3 × 10 min. The antibody-bound target proteins were detected using ECL system (Millipore, Billerica, MA, USA). Quantification of the band intensities was carried out with Gel Image System ver. 4.00 software (Tanon, Shanghai, China). GAPDH (Cell Signaling Technology, Danvers, MA, USA) was evaluated as an internal reference.

**Statistical Analysis**

Data were processed with SPSS software (IBM Statistics for Windows, Version 20.0; IBM Corp., Armonk, NY, USA). Data were expressed as mean ± standard deviation (±). Differences in data between groups were analysed for statistical significance by a paired-test, one-way analysis of variance (ANOVA) followed by a Turkey post-hoc test, or single-factor ANOVA followed by a least significant difference test. Values of $P < 0.05$ were considered statistically significant.

**RESULTS**

**Blood pressure**

The SBP findings are shown in Figure 1. SBP in the model group was significantly higher than that in the Normal group ($P < 0.01$). After the 30-day intervention, SBP in the EA and Losartan groups was significantly lower than that in the model group ($P < 0.01$).

**Left ventricular mass index and echocardiographic parameters**

The findings for LVMI and echocardiographic parame-
Relatively small myocardial cells in terms of cross-section deeply stained. The EA and Losartan groups showed cell spacing was wider, and the nucleus was enlarged and redded: the cross-sectional area of the cells was larger, the cardiac cells in the model group was relatively disordered with the normal group, the arrangement of myocardial cells was significantly decreased (P < 0.01), while the relative gene expression levels of PTEN and ANP in the EA group was significantly decreased (P < 0.01).

The HE staining results are shown in Figure 3. Compared with the normal group, the model group had significantly lower relative gene expression levels of PI3K, Akt, and mTOR (P < 0.01), and significantly higher relative gene expression levels of PTEN and ANP (P < 0.01). Compared with the model group, the relative gene expression levels of PI3K, Akt, and mTOR in the EA and Losartan groups were significantly increased (P < 0.01), while the relative gene expression levels of PTEN and ANP in the EA group was significantly decreased (P < 0.01). The ratios of phosphorylated and non-phosphorylated proteins are shown in Figure 5. Compared with the normal group, the model group had significantly lower ratios of p-PI3K/t-PI3K, p-Akt/t-Akt, and p-mTOR/t-mTOR (P < 0.01), and significantly higher ratios of p-PTEN/t-PTEN (P < 0.01). The EA and Losartan groups had significantly higher ratios of p-PI3K/t-PI3K, p-Akt/t-Akt, and p-mTOR/t-mTOR (P < 0.01) and significantly lower ratios of p-PTEN/t-PTEN (P < 0.01) compared with the model group.

**DISCUSSION**

According to the preventive treatment philosophy of traditional Chinese medicine, it is advisable to prevent a disease before it occurs, and if not possible, to treat the disease before it progresses and its effects worsen. Myocardial hypertrophy, a recognised symptom of hypertension-related target organ damage, is a strong and independent risk factor for adverse cardiovascular outcomes like coronary heart disease and heart failure. Therefore, clarification of the mechanisms for myocardial hypertrophy during the development of hypertension may provide crucial information on how to prevent cardiovascular disease from occurring, and how to intervene as quickly as possible. According to the theory of traditional Chinese medicine, the aetiology of hypertensive myocardial hypertrophy is mainly caused by disorder of Qi and imbalance of Yin and Yang. Thus, acupoints Taichong (LR 3) and Baihui (DU 20) were evaluated in the present study. Furthermore, Taichong (LR 3) and Baihui (DU 20) are the two most commonly used acupoints in the treatment of hypertension and myocardial hypertrophy in humans, and both can help to lower blood pressure and reverse myocardial hypertrophy. In this study, we investigated the anti-hypertensive and anti-hypertrophic effects of EA at Taichong (LR 3) and Baihui (DU 20) on SHRs. The results confirmed that SHRs had significantly higher caudal artery SBP than normal control rats, and that a 30-day treatment with EA could inhibit the blood pressure rise in SHRs and

Table 2 Amplification conditions

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even lower their blood pressure to some extent. The results further showed that the model group had significantly increased LVMI, LVP-WT, and IVST and significantly decreased LVEDD and LVEF, indicating a clear tendency toward left ventricular hypertrophy. Compared with the model group, the EA group showed significant improvement, suggesting that EA at Taichong (LR 3) and Baihui (DU 20) can effectively lower blood pressure and inhibit the development of hypertensive myocardial hypertrophy. These findings are consistent with previous research.

**Figure 2** LVMI and echocardiographic parameters (\( \bar{x} \pm s, n = 12 \))

- Normal group: untreated Wistar Kyoto rats;
- Model group: untreated spontaneously hypertensive rats;
- EA group: spontaneously hypertensive rats treated with electroacupuncture at Baihui (DU 20) and Taichong (LR 3);
- Losartan group: spontaneously hypertensive rats administered losartan at 30 mg/kg per day.

- A: left ventricular mass index (LVMI);
- B: left ventricular ejection fraction (LVEF);
- C: end-diastolic interventricular septal thickness (IVST), left ventricular end-diastolic dimension (LVEDD), and left ventricular posterior wall thickness (LVP-WT).

**Figure 3** Hematoxylin and eosin staining of left ventricular tissue (× 400)

- Normal group: untreated Wistar Kyoto rats;
- Model group: untreated spontaneously hypertensive rats;
- EA group: spontaneously hypertensive rats treated with electroacupuncture at Baihui (DU 20) and Taichong (LR 3);
- Losartan group: spontaneously hypertensive rats administered losartan at 30 mg/kg per day.

**Figure 4** Gene expression levels of PI3K, Akt, mTOR, PTEN, and ANP (\( \bar{x} \pm s, n = 6 \))

- Normal group: untreated Wistar Kyoto rats;
- Model group: untreated spontaneously hypertensive rats;
- EA group: spontaneously hypertensive rats treated with electroacupuncture at Baihui (DU 20) and Taichong (LR 3);
- Losartan group: spontaneously hypertensive rats administered losartan at 30 mg/kg per day.

- PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; mTOR: mammalian target of rapamycin; PTEN: tensin homolog deleted on chromosome ten; ANP: atrial natriuretic peptide.
with those in above studies. The PI3K/Akt signal transduction pathway is closely related to cellular activities and involved in the processes of cell growth, differentiation, survival, apoptosis, and malignant transformation by mediating many external stimulus signals that induce these processes. The PI3K/Akt signalling pathway was reported to play a key role in cardiac development and postnatal growth. Furthermore, short-term activation of Akt can inhibit cardiac myocyte apoptosis, improve myocardial remodelling, and control cardiac hypertrophy. The function of activated Akt in promoting cell growth and survival is mainly achieved by expression of its effector, mTOR. mTOR is involved in the determination of cell size and organ morphology, and is known as the ‘central regulator’ of cells. mTOR also has an important role in regulating the development of myocardial hypertrophy. PTEN is a negative regulator of the PI3K/Akt/mTOR signalling pathway, and can weaken the signal transduction of PI3P, thereby reducing the activation of Akt and preventing all downstream signal transduction events regulated by Akt. ANP is a response index for cardiomyocyte hypertrophy, and is also known as cardiac hypertrophy-specific factor. A previous study showed that the ANP mRNA expression levels in the atrium and ventricle of SHRs were 50%-70% higher than those in normal rats, and that these mRNA contents increased with age and blood pressure. In the present study, the expression levels of PI3K, Akt, and mTOR in the myocardial tissue were significantly lower in the model group compared with the normal group, while the expression levels of PTEN and ANP were significantly higher. Furthermore, EA at Taichong (LR 3) and Baihui (DU 20) significantly increased the expression levels of PI3K, Akt, and mTOR, while decreasing the expression levels of PTEN and ANP in the myocardial tissue of SHRs, suggesting that EA at these two acupoints can inhibit the development of hypertensive myocardial hypertrophy by activating the PI3K/Akt/mTOR signalling pathway.

In conclusion, our findings suggest that EA at Taichong (LR 3) and Baihui (DU 20) inhibited the development of myocardial hypertrophy and improved the cardiac function of SHRs, possibly through regulation of the PI3K/Akt/mTOR signalling pathway.

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